MONOCLONAL ANTIBODY-BASED ELISA FOR THE DETECTION OF CIRCULATING *ENTAMOEBA HISTOLYTICA* ANTIGEN IN HEPATIC AMEBIASIS IN HAMSTERS (*MESOCRICETUS AURATUS*)

Nitaya Thammapalerd[1], Jeevan Bahadur Sherchand[1], Daroon Kotimanusvanij[2], Thaiyooth Chintana[3] and Savanat Tharavanij[1]

[1]Department of Microbiology and Immunology, [3]Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400; [2]Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Abstract. Circulating amebic antigens were determined by using a sandwich ELISA with specific monoclonal antibody in the sera of 35 group I hamsters, 7 of which were sacrificed at intervals after hepatic inoculation with 500,000 axenically grown HM:1:IMSS strain of *E. histolytica* trophozoites, 7 group II infected hamsters in which metronidazole treatment was given and 18 group III uninfected controls. Amebic antigenemia was demonstrated in 5 of 7 (71.4%), 6 of 7 (85.7%), 7 of 7 (100%), 7 of 7 (100%) and 7 of 7 (100%) of group I hamsters on days 5, 10, 15, 20 and 30 of infections respectively, whereas 6 of 7 (85.7%) of group II hamsters were weakly positive, one was negative and all 18 group III hamsters were negative. The sensitivity of the assay was 100% after the animals were infected 15 days onwards. The level of antigenemia in hamsters of group I with abscess was significantly higher than those of the same group without abscess ($p < 0.05$). Absence or reduction of antigenemia after treatment could be interpreted to mean a positive test of cure and favorable therapeutic response. The MAb-PAb-based ELISA for the detection of circulating *E. histolytica* represents a simple and sensitive diagnostic test for invasive amebiasis in hamsters. Application of this test in amebic liver abscess patients should be of diagnostic value for indication of present infection or test of cure after successful treatment.

INTRODUCTION

Amebiasis, a disease caused by *Entamoeba histolytica* with or without clinical symptoms, occurs throughout the world, especially in tropical and subtropical climates and in places with poor sanitary conditions due to fecal contamination of water and food or direct fecal-oral contact. Serological tests such as immunoelectrophoresis (IEP), cellulose acetate membrane precipitin (CAP) test and enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *E. histolytica* are particularly useful for the diagnosis of extraintestinal amebiasis such as amebic liver abscess (Tharavanij and Chaicumpa, 1969; Thammapalerd *et al*, 1981; Samrejrongroj and Tharavanij, 1985; Nicholls *et al*, 1994). In such cases stool examination is often negative and positive antibody titers may persist for months or years after successful treatment. Therefore, it is difficult to differentiate between present and past infections (Sepulveda and Martinez-Palomo, 1982). Detection of circulating amebic antigens would be better correlated with present infection. Circulating amebic antigens may be in the form of immune complexes, the detection of which have been documented by using counterimmunoelectrophoresis (CIEP), enzyme-linked immunosorbent assay (ELISA), radioimonunoassay (RIA) with polyclonal antibodies (PAb) and C1q solid phase assay (Mahajan and Ganguly, 1980, Vinayak *et al*, 1986, Pillai and Mohimen, 1982, Gandhi *et al*, 1988; Mukherjee *et al*, 1994; Nuti *et al*, 1982). Prompted by the success in the application of MAb-based ELISA, whereby the Eh208C2-2 MAb was used in the detection of amebic antigens in the feces of all 13 patients haboring *E. histolytica* trophozoites (Thammapalerd and Tharavanij, 1991; Wonsit *et al*, 1992) and intrahepatic localization of trophozoite antigens by MAb-based IFA and MAb-based immunoperoxidase (IPx) tests (Sherchand *et al*, 1994), we have extended this study to detect circulating amebic antigens in a hamster model after intrahepatically inoculation with *E. histolytica* trophozoites.

Correspondence: Nitaya Thammapalerd.
Fax: 66 2 246 8340; Email: tmntm@mahidol.ac.th

MATERIALS AND METHODS

Amebic and culture conditions

The HM1 : IMSS strain of *E. histolytica* was maintained axenically with twice weekly sub-culture in screw-capped tubes in TPS-1 medium in the presence of 10% heat-inactivated bovine serum and vitamin mixture according to the technique described by Diamond (1968).

Preparation of *E. histolytica* antigen for animal inoculation

Log-phase (72 hours) cultures of trophozoites were harvested by chilling on ice for 5 minutes, followed by centrifugation at 350*g* for 5 minutes, counted on a hemacytometer, resuspended in a volume of complete TPS-1 medium to give a final concentration of 2.5×10^5 trophozoites per 100 µl. Tubes containing amebae were kept on ice for no longer than 10 minutes before inoculation.

Experimental animals

Male syrian golden hamsters (*Mesocricetus auratus*), 50-60 days old were kindly supplied by the animal house of the Faculty of Tropical Medicine, Mahidol University and the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. BALB/c mice were kindly supplied by AFRIMS and adult New Zealand white rabbits aged 2 to 3 months were purchased from the Faculty of Veterinary Medicine, Chulalongkorn University.

Surgical and hepatic inoculation procedure

The hamsters were fasted for 18 hours and received intraperitoneal injections of sodium pentabarbital solution (42 mg/kg body weight). Their abdominal cavities were opened by longitudinal midline incision of approximately 3 cm in length, the liver was exposed, and the inoculum was slowly injected into the left lobe of the exposed liver. After inoculation, care was taken to preclude leakage and hemorrhage of the inoculum by blotting the area with sterile cotton swabs. The abdominal wall and skin were satured separately with 0-3 normal catgut. Aseptic precautions were observed through out each operation which took about 10 minutes to complete. After operation, the animals were housed and fed on commercial mouse diet, vegetables and water *ad libitum* until sacrified. Sixty male hamsters were divided into three groups. Group I comprising 35 animals, each animal was inoculated intrahepati-cally with 500,000 trophozoites of *E. histolytica* in 0.2 ml of sterile phosphate buffered saline (PBS) solution, pH 7.2 using disposable tuberculin syringe attached to a 1 cm 26 gauge needle. The abdominal wall and skin were satured separately with 3-0 sterile normal catgut. Group II comprising 7 animals, they were inoculated with the same dose as those animals in Group I and 20 days post inoculation, they were treated with metronidazole (200 mg/kg body weight) by oral gavage for 6 consecutive days and examined 10 days after the last dose of treatment. Group III comprising 18 age-matched control animals, 15 of which were similarly given 0.2 ml of sterile PBS, while 3 were not given any injection.

Sera

Blood was collected from the heart of the hamsters prior to intrahepatic inoculation with *E. histolytica* and on days 5, 10, 15, 20 and 30 days post-inoculation with *E. histolytica* of group I animals, 36 days of group II animals and 30 days of group III animals, respectively. Sera were separated and protease inhibitors were added at a final concentration of 1 mM of phenyl methyl-sulphonyl-fluoride (PMSF) and 1 mM of n-1-p-tosyl-L-lysine chloromethyl ketone (TLCK), the sera aliquoted in 1 ml portion and kept frozen at -78°C until tested.

Preparation of rabbit anti-*E. histolytica* polyclonal antibodies (PAbs)

Two healthy rabbits were immunized intramuscularly five times with 0.5 ml of crude amebic whole cell lysate equivalent approximately to 5×10^6 trophozoites of the HM1:IMSS strain of *E. histolytica* emulsified with an equal volume of Freund's complete adjuvant initially and Freund's incomplete adjuvant subsequently at an interval of two to three weeks. The rabbits were bled by cardiac puncture 9 days after the last immunizing dose, and the serum IFA antibody titers against HM-1:IMSS were both 1:640. Sera were aliquoted and kept at -20°C until used. Serum IgG was purified by affinity chromatography on protein A Sepharose CL-4B according to the technique of Seppalla *et al* (1981). The purified polyclonal IgG (PIgG) at the concentration of 5 µg/ml gave IFA antibody

titer of 1:1,280. The PIgG was aliquoted, lyophilized and stored at -78°C until used.

Anti-*E. histolytica* MAb

Murine MAbs were raised against the HM1:IMSS strain of *E. histolytica* in Sp2/0 myeloma cells according to the method of Galfre and Milstein, 1982 (Thammapalerd and Tharavanij, 1991). Several antibody-secreting hybridoma clones were obtained, one in particular, and IgG1 specific Eh208C2-2 MAb was selected for this study. The IgG fraction of the ascites (MIgG) was purified by protein A Sepharose CL-4B affinity chromatography as described above and used in a sandwich ELISA system for the detection of circulating antigens in the sera of hamsters with hepatic amebiasis, infected hamsters after treatment and healthy hamsters. The protein content was determined by the method of Lowry *et al* (1951) using bovine serum albumin (Sigma Chemical Company, St Louis, USA) as standard.

MAb-PAb-based ELISA for the detection of *E. histolytica* antigen in the sera

The technique used followed antibody sandwich ELISA previously described by Wonsit *et al* (1992). Briefly, a 96-well polystyrene micro ELISA plate (Dynatech) was coated with 100 µl of 10 µg/ml of Eh208C2-2 MIgG at 37°C for 1 hour and further at 4°C overnight. The unbound components were washed twice with distilled water each for three minutes. The nonreactive sites were blocked with PBST containing 5% nonfat dry milk (Carnation) at 37°C for 1 hour. After washing 4 times with PBST, 100 µl of sera from infected, control and drug-treated hamsters at various days post inoculation of *E. histolytica* was added to each well followed by incubation at 37°C for 2 hours. The plate was again washed with PBST and 100 µl of 10 µg/ml of PIgG added, followed by incubation at 37°C for 1 hour. After washing, 100 µl of goat alkaline phosphatase (AP) - conjugated anti-rabbit IgG (Zymed) at 1:1,000 dilution in PBST-1% BSA was added to each well, followed by incubation at 37°C for 1 hour. The plate was washed and 100 µl of substrate solution (p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8) added. The enzyme-substrate color reaction was allowed to develop at room temperature and then stop with 3N NaOH after 40 minutes. The optical density (OD) of each well was measured at 405 nm with an ELISA plate reader (Titertek Multiskan, MCC/340, Flow Laboratories).

The optimal dilutions of rabbit antisera and conjugate were determined using a checkerboard titration. For each test, a negative, a positive and a PBST controls were included. Each serum sample was tested in duplicate and the mean OD value calculated.

Statistical analysis

Continuous variables were compared using the nonparametric Kruskal-Wallis method. All p values less than 0.05 were considered to indicate statistical significance.

RESULTS

Mab-PAb-based ELISA for the detection of circulating *E. histolytica* antigens

The cut-off OD value of 0.257 was based on the mean +2SD of reactivity in normal hamsters. Therefore, the test sample was considered positive if its OD value was equal to or above 0.257.

Amebic antigenemia was demonstrated in 5 of 7 (71.4%), 6 of 7 (82.7%), 7 of 7 (100%), 7 of 7 (100%) and 7 of 7 (100%) of group I hamsters on days 5, 10, 15, 20 and 30 of infections respectively, whereas 6 of 7 (85.7%) of group II hamsters were weakly positive and one (14.3%) was negative and all 18 of group III hamsters were negative (Fig 1).

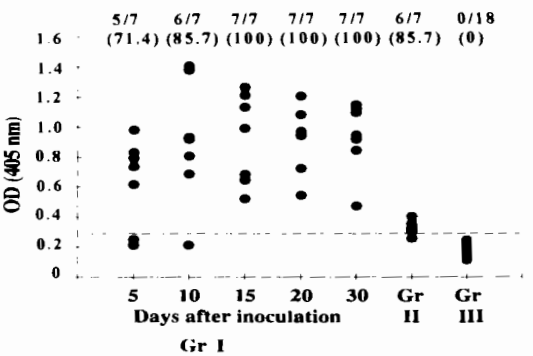

Fig 1–Scatter diagram of the optical density (OD) using MAb-PAb-based ELISA for the detection of circulating *E. histolytica* antigens of 3 groups of hamsters at various days post inoculation.
Group I, 35 animals intrahepatically infected with 5×10^5 trophozoites of *E. histolytica* Group II, 7 infected and treatment group and, Group III, 18 age-matched control group.

In group I hamsters, abscess were demonstrated either by gross pathology or by H & E staining in 3 (42.8%), 3 (42.8%), 2 (28.6%), 4 (57.1%) and 3 (42.8%) hamsters on days 5, 10, 15, 20 and 30 respectively. No obvious abscess was seen in group II hamsters receiving metronidazole treatment. Livers of hamsters in group III were all normal. The level of antigenemia in hamsters of group I with abscess was higher than hamsters of the same group without abscess ($p < 0.05$) (Fig 2A, 2B). Such difference was not correlated with days post inoculation ($p > 0.05$).

DISCUSSION

The golden hamster (*Mesocricetus auratus*) is the animal of choice for the study of hepatic amebiasis and for the detection of circulating amebic antigens in our laboratory whereby liver tissue sections containing *E. histolytica* trophozoites were recognized by MAb-based IFA and MAb-based IPx tests (Sherchand *et al*, 1994). Wide necrotic areas develop in the liver after trophozoites are inoculated right into the organ. These lesions respond well to the standard antiamebic therapy with metronidazole (Flagyl 200 mg/kg body weight).

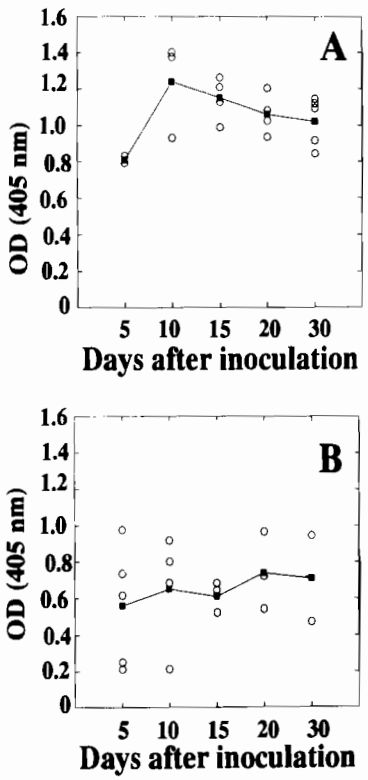

Fig 2–Means OD values of ELISA for the detection of circulating *E. histolytica* antigens with liver abscess (A) and without liver abscess (B) of hamsters after various days post inoculation with *E. histo-lytica* trophozoites.

A number of tests has been employed to detect parasitic antigens other than amebic antigens in sera of infected humans and murines; however, publications on the detection of circulating amebic antigens in human sera and pus, and in experimental amebiasis are scanty. Detection of antigen in human liver pus and sera using specific antiserum raised in rabbits against axenic amebic antigen and CIEP by Mahajan and Ganguly (1980) gave 92% (115/125) and 27% (24/89) positivity respectively in patients with amebic liver abscess. Vinayak *et al* (1986) could detect amebic antigen in circulating immune complexes (CIC) in 93% of the confirmed cases of amebic liver abscess using polyclonal antibodies and ELISA. Onyemelukwe and Onyewotu (1981) reported the presence of immune complex activity in 58% (7 of 12) of amebic liver abscess patients in Nigeria in which all these patients were positive by amebic gel diffusion test. However, they did not show that specific amebic antigens are present in those intact immune complexes.

The present study demonstrated that the double antibody sandwich ELISA is potentially useful for amebic antigen detection in sera of hepatic hamsters experimentally infected with *E. histolytica* trophozoites with the sensitivity of 100% after 15 days onwards of infection. Circulating amebic antigens were detected as early as day 5 throughout the experimental period of 30 days post inoculation in infected group I hamsters and remained positive thereafter, whereas 6 of 7 (85.7%) of drug treated groups II hamsters were weakly positive and one (14.3% was negative and all 18 of age-matched control group III hamsters were negative. In group II of infected and drug-treated animals, 20 days post inoculation, drug were given orally for 6 consecutive days and 10 days after the last dose of treatment sera were tested. If drug was given earlier than 20 days post inoculation with *E. histolytica* or if follow up for more than 30 days were carried out, it is speculated that the assay would become negative in all metronidazole-treated hamsters. If this turns out to be the case, this assay may be used as a test of cure and will have practical

value in distinguishing between present and past infection in patients with invasive amebiasis. In addition, attempts to develop a more sensitive and simple antigen detection system, such as a dot-blot ELISA using nitrocellulose paper should be evaluated.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Faculty of Tropical Medicine, Mahidol University. Eh208C2-2 MAb used in this study was produced with the support from the USAID/PSTC Program, Grant No. 936-5542-G-00-6026-00. We are grateful to Professor Gordon B Bailey, Morehouse School of Medicine, Atlanta, Georgia for the HM-1:IMSS strain of *E. histolytica*.

REFERENCES

Diamond LS. Techniques of axenic cultivations of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amoeba. *J Parastiol* 1968; 54 : 1047-56.

Gandhi BM, Irshad M, Acharya BK, Tandon BN. Amebic liver abscess and circulating immune complexes of *Entamoeba histolytica*. *Am J Trop Med Hyg* 1988; 39 : 440-4.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193 : 265-75.

Mahajan RC, Ganguly NK. Amoebic antigen in immunodiagnosis and prognosis of amoebic liver abscess. *Trans R Soc Trop Med Hyg* 1980; 74 : 300-2.

Mukherjee RM, Maitra TK, Mohimen A, Mehra S, Jalan KN. Detection of immune complexes in tissues of persons infected with *Entamoeba histolytica*. *Trans R Soc Trop Med Hyg* 1994; 88 : 546-7.

Nicholls RS, Restrepo MI, Duque S, Lopez MC, Corredor A. Standardization and evaluation of ELISA for serodiagnosis of amoebic liver abscess. *Mem Inst Oswaldo Cruz* 1994; 89 : 53-8.

Nuti M, D'Amelio R, Seminara R, Palmisano L, Aiuti F. Circulating immune complexes in amoebiasis. *Trans R Soc Trop Med Hyg* 1982; 76 : 567-8.

Onyemelukwe GC, Onyewotu II. Amoebic liver abscess-serum anticomplementary screening for immune complexes. *Trans R Soc Trop Med Hyg* 1981; 75 : 613-4.

Pillai S, Mohimen A. A solid-phase sandwich radioimmunoassay for *Entamoeba histolytica* proteins and the detection of circulating antigens in amoebiasis. *Gastroenterology* 1982; 83 : 1210-6.

Samrejrongroj P, Tharavanij S. Assessment of validity of counterimmunoelectrophoresis and ELISA in the routine diagnosis of amebiasis. *Southeast Asian J Trop Med Public Health* 1985; 16 : 365-70.

Seppalla I, Sarvas H, Peterfy F, Makela O. The four subclasses of IgG can be isolated from mouse serum by using protein- A-Sepharose. *Scand J Immunol* 1981; 14 : 335-42.

Sepulveda B, Martinez-Palomo A. The immunology of amoebiasis produced by *E. histolytica*. In : Cohen S, Warren KS, eds. Immunology of Parasitic Infections. Oxford: Blackwell Scientific Publications, 1982; 170-91.

Sherchand JB, Thammapalerd N, Riganti M, Tharavanij S, Punpoowong B. Monoclonal antibody-based immunohistochemical demonstration of *Entamoeba histolytica* in liver tissue of experimentally infected hamster (*Mesocricetus auretus*). *Int J Parasitol* 1994; 26 : 909-16.

Thammapalerd N, Tharavanij S. Biomedical applications of monoclonal antibodies (McAb) against *Entamoeba histolytica*. In: Maramorosch K. ed. Biotechnology for Biological Control of Pests and Vectors. Florida: CRC Press. 1991; 163-78.

Thammapalerd N, Tharavanij S, Samrejrongroj P. Cellulose acetate membrane precipitin (CAP) test in amebiasis using antigens from four different strains of *Entamoeba histolytica*. *Southeast Asian J Trop Med Pub Health* 1981; 12 : 353-6.

Tharavanij S, Chaicumpa W. Immunoelectrophoresis test for amoebiasis. *Bull WHO* 1969; 40 : 343-53.

Vinayak VK, Purnima, Singh, K, Venkatwswarlu K, Nain CK, Mehta SK. Specific circulating immune complex in amoebic liver abscess. *J Clin Microbiol* 1986; 23 : 1088-90.

Wonsit R, Thammapalerd N, Tharavanij S, Radomyos P, Bunnag D. Enzyme-linked immunosorbent assay based on monoclonal and polyclonal antibodies for the detection of *Entamoeba histolytica* antigens in faecal specimens. *Trans R Soc Trop Med Hyg* 1992; 86 : 166-9.